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Precipitation methods for high-density lipoprotein cholesterol measurement compared, and final evaluation under routine operating conditions of a method with a low sample-to-reagent ratio

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We evaluated six precipitation methods for high-density lipoprotein cholesterol (HDL-chol) determination: the heparin/Mn²⁺ precipitation reagent method (Hep), two variants of the phosphotungstic acid/Mg²⁺ method (Tung-L and Tung-B), the dextran sulfate 50 000/Mg²⁺ method (Dex), the PEG 6000 method (PEG), and the PEG 6000/dextran sulfate 15 000 (PEG/Dex) method. The Tung-B and PEG/Dex precipitation methods have a low sample/precipitation reagent volume ratio (<0.4). The Tung-B, Dex, PEG, and PEG/Dex methods gave similar values, averaging within 0.1 mmol/L of each other, showing that the precipitation selectivity of these methods is comparable. The precipitation efficiency of Tung-B and PEG/Dex, however, was superior. Ultrafiltration of the supernatants was needed only at triglyceride concentrations >16.4 mmol/L (undiluted sample) or >28.0 mmol/L (sample diluted twofold); however, ultrafiltration without dilution was the most accurate method. Results of Tung-B under routine conditions (33 technicians) agreed well with those of the PEG method for 406 normo- and hyperlipidemic plasma samples. By comparison with the HDL-chol method from the Centers for Disease Control and Prevention, the Tung-B method showed a total error of 10.6%, which fulfills the criteria of the National Cholesterol Education Program for HDL-chol analysis. In conclusion, with motivated personnel, Tung-B is a reliable, cost-effective method for routine HDL-chol analysis.

INDEXING TERMS: lipoproteins • method comparison • laboratory management

Proper risk estimation for coronary heart disease involves total cholesterol and high-density lipoprotein cholesterol (HDL-chol) measurements [1–4].³ HDL-chol measurement is still problematic, despite the general use of a Proposed Selected Method [5], especially in samples that have been frozen and in lipemic plasma samples [6, 7]. Earlier, we introduced and fully validated the polyethylene glycol (PEG) 6000 method [8]. Given its excellent agreement with ultracentrifugation/heparin-Mn²⁺ (Hep) precipitation and its problem-free use in all our research projects in the last 15 years, the PEG 6000 method has, in our area, gradually gained the status of a “Regional Designated Comparison Method.” This attainment was stimulated by its superior precipitation efficiency among all the first-generation precipitation methods [6, 9]. Although the results obtained with the PEG 6000 method are scientifically reliable, the analyses must be performed by a specialized group of technicians.

Given the steady growth in the number of requests for HDL-chol determinations, we felt the need for another method, one that could be used more generally, i.e., in the routine laboratory also. For this purpose we evaluated several “first-generation” precipitation methods, includ-

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Received June 5, 1996; revised November 14, 1996; accepted November 15, 1996.

³ Nonstandard abbreviations: chol, cholesterol; Hep, heparin/Mn²⁺; Tung-L, phosphotungstate/Mg²⁺ (Lopes-Virella version); Tung-B, phosphotungstate/Mg²⁺ (Boehringer version); Dex, dextran sulfate 50 000/Mg²⁺; PEG 6000, polyethylene glycol 6000; PEG/Dex, polyethylene glycol 6000/dextran sulfate 15 000; first- (second-) generation precipitation method, HDL assay performed with concentrated (diluted) precipitation reagent, resulting in concentrated (diluted) HDL samples; macro- and micromethods, cholesterol analysis under conditions optimal for cholesterol concentrations between 0.1 and 3.5 mmol/L or from 0.01 to 0.35 mmol/L, respectively; CDC, Centers for Disease Control and Prevention.

ing the Proposed Selected Method, which uses dextran sulfate 50 000/Mg²⁺ (Dex) [5]. We also included two "second-generation" precipitation methods, which use a more diluted precipitation reagent to obtain more-effective precipitation. In this study we clearly show that not only methodological but also personnel aspects must be taken into account in generating the best analytical results in a routine laboratory.

Materials and Methods

PROCEDURES, PART 1

In the first part of this study, the merits of six different precipitation methods were compared by a single experienced technician. The first-generation procedures used are listed here in the same order as in our previous study [9]. In that study these procedures were validated and compared against an ultracentrifugation/precipitation procedure, very similar to the Centers for Disease Control and Prevention (CDC) HDL method, except that the cholesterol was determined with enzymatic analysis. In these first-generation HDL methods, the volume of serum is >5 times the volume of precipitation reagent. In Tung-B and PEG/Dex, second-generation methods, the volume of serum is <0.4 times the volume of precipitation reagent. In the first part of the study, in which only a limited number of samples were analyzed, the results were compared with consensus values, i.e., the average values obtained with four different methods that appeared to give very similar results. Assay conditions of the various precipitation methods, and of the different ways we analyzed for cholesterol, are summarized in Table 1.

In the first part of this study, the following precipitation procedures were evaluated:

Hep [9, 10]. One milliliter of serum was mixed with 100 μ L of a mixture of equal parts of 40 g/L sodium heparinate solution (156 USP units/mg; Organon, Oss, The Netherlands) and 1 mol/L MnCl₂ solution. After 10 min, the samples were centrifuged for 15 min at 4000g. The supernate was aspirated as described [9] with a Pasteur pipette. When turbid, the supernatant was filtered through a 0.20- μ m (average pore size) filter (FP030/3; Schleicher and Schüll, Dassel, Germany) [9]. Cholesterol content was quantified with the macromethod described in Table 1, and the final concentration was corrected for dilution.

Tung-L method: phosphotungstate/Mg²⁺ [9–11]. Optimized phosphotungstate reagent was prepared by dissolving 40 g of phosphotungstic acid (cat. no. 583; Merck, Darmstadt, Germany) in distilled water. The pH of the solution was adjusted to 6.15 with 1 mol/L NaOH [12], and the reagent was diluted to 1 L with water. The Mg²⁺ concentration of the 2.5 mol/L MgCl₂ solution was checked by atomic absorption spectrometry [13]. One milliliter of serum was mixed with 20 μ L of the 2.5 mmol/L MgCl₂ solution (final concentration: 1.39 μ mol of phosphotungstic acid and 50 μ mol of MgCl₂ per milliliter of serum).

Table 1. Procedures used in the two parts of this study.

First part		Second part	
Precipitation methods	Cholesterol method	Precipitation methods	Cholesterol method
Heparin/Mn ²⁺	Macro ^a		
Phosphotungstate/Mg ²⁺ (Tung-L)	Macro ^a		
Phosphotungstate/Mg ²⁺ (Tung-B)	Micro ^b	Tung-B	Micro ^c
Dextran sulfate/Mg ²⁺	Macro ^a		
PEG 6000	Macro ^a	PEG 6000	Macro ^a
PEG/Dex	Micro ^b		

^a Macro method on the Multistat IL-III analyzer with the use of CHOD-PAP reagent (Boehringer Mannheim, cat. no. 237574). Sample/reagent volumes (μ L): 5/150, diluted further with 45 μ L of rinsing water; calibrated with Preciset solution diluted with Brij-35 wetting agent (no. 430AG-6; Sigma Chemical Co., St. Louis, MO) to final cholesterol concentrations of 0.08, 0.39, and 1.29 mmol/L. The method for total cholesterol measurement regularly checked for accuracy against the CDC-certified Abell-Kendall method (Lipid Reference Laboratory, Rotterdam) in the framework of a specific Dutch cholesterol standardization program gave observed biases <1.2%.

^b Micro method on the Multistat IL-III analyzer as above but with sample/reagent volumes (μ L) of 80/80 further diluted with 20 μ L of rinsing water; calibrated with Preciset diluted with Brij to final cholesterol concentrations of 0.130 and 0.388 mmol/L.

^c Micro analysis on the Hitachi 747 analyzer with CHOD-PAP reagent (cat. no. 1489704). Sample/reagent volumes (μ L): 20/250; calibrated with calibrating serum (Boehringer, cat. no. 759350) and the specific Hitachi calibrating procedure. The method is regularly checked for accuracy of total cholesterol measurement against the CDC-certified Abell-Kendall procedure as mentioned above; observed biases thus far have been <1.3%. Imprecision = 1.9% (n = 30). To attain this accuracy, we routinely corrected the target value of the calibrating serum (provided by the supplier) by -4%.

After 15 min, the HDL fraction was isolated as described, and cholesterol was determined with the macromethod.

Tung-B method: phosphotungstate/Mg²⁺. We also applied a second version of the phosphotungstate/Mg²⁺ method, using reagent from Boehringer Mannheim (Mannheim, Germany; cat. no. 543004): 200 μ L of serum was mixed with 500 μ L of precipitation reagent (final concentrations: 1.1 μ mol of phosphotungstic acid and 50 mmol of MgCl₂ per milliliter of serum). After incubation, the supernate was isolated as described above, and the cholesterol was determined with a microassay (see Table 1).

Dex [5]. We used reagent obtained from Johnson & Johnson (Rochester, NY), pipetting 0.5 mL of serum into the Ektachem (Johnson & Johnson) HDL assay tube containing dextran sulfate 50 000/magnesium reagent. After incubation, we applied the conditions for fractionation and cholesterol analysis described above. One milliliter of serum was mixed with 100 μ L of a mixture of equal parts of 2 mol/L MgCl₂ and dextran sulfate; the HDL fraction was isolated as described and assayed for cholesterol with the macromethod.

PEG 6000 [6, 8]. One milliliter of serum was mixed with 200 μ L of PEG 6000 solution [22.5 g of Merck no. 807491

and 22.5 g of Fluka (Fuchs, Switzerland) no. 81260, both dissolved in 100 mL of 0.2 mol/L Tris-HCl buffer, pH 8.2]. The final PEG 6000 concentration in serum was thus 75 g/L. After thorough vortex-mixing, incubation, and centrifugation, HDL fractions were isolated as above and were determined for cholesterol with the macromethod.

PEG/Dex [14]. The reagent, from Instruchemie (Hilverum, The Netherlands), contained 0.15 mol/L NaCl, 100 g/L PEG 6000, 37.4 mg/L dextran sulfate 15 000, and 2.6 mmol/L MgCl_2 . Serum (100 μL) was mixed with 1000 μL of the HDL-chol precipitation reagent. After incubation, the supernate was isolated and cholesterol was assayed with the micromethod.

Cholesterol quantification. The cholesterol in the supernates was determined with a Multistat IL-III (Instrumentation Laboratory, Lexington, MA) centrifugal analyzer by one of two methods, depending on the final concentration of the analyte in the sample after dilution (Table 1). We used the CHOD-PAP reagent (cat. no. 237574; Boehringer Mannheim). The results of both the micro- and the macro-version assays (for 1.2- and 11-fold-diluted plasma samples, respectively) showed good agreement ($r = 0.999$). In all measurements, the actual cholesterol concentration found for the HDL fractions was corrected for the "precipitation blank" (apparent cholesterol concentration of appropriately diluted precipitation reagent); this generally amounted to <0.03 mmol/L, except for the Hep reagent, which gave a value of 0.10–0.15 mmol/L.

Triglycerides assay. Triglycerides were analyzed on the Hitachi 747 analyzer with reagent no. 1361155 (both from Boehringer Mannheim). We checked the method's accuracy against that of a semiautomated colorimetric method [15]. Imprecision was 1.6% ($n = 30$) for concentrations ≤ 12 mmol/L.

PROCEDURES, PART 2

In the second part of this study, the Tung-B method was evaluated under routine conditions by 33 different technicians in the routine laboratory, the technicians having been individually instructed by the one experienced colleague who performed the first part of the study. Because of the extensive previous validation and experience with the PEG 6000 method, we used this method for comparison. Cholesterol was measured in the routine laboratory with a Hitachi 747 analyzer (using the microassay version described in Table 1). As a further validation of the Tung-B method, we analyzed for 3 days in duplicate 7 fresh serum samples with HDL-chol concentrations ranging from 0.73 to 2.13 mmol/L; aliquots of these sera were also analyzed by the official CDC HDL-chol method at the Lipid Reference Laboratory (Rotterdam, The Netherlands) under the supervision of C. Cobbaert-Boersma.

SPECIMENS

Blood samples from normolipidemic subjects and from patients with various types of hyperlipoproteinemia were drawn into Vacutainer Tubes (Becton Dickinson, Rutherford, NJ). Serum was isolated within 2 h and was stored at 4 °C for no more than 2 days. In the first part of the study, 26 fresh serum samples with triglycerides <4.7 mmol/L were analyzed. In the second part, 406 sera or EDTA-containing plasma samples were analyzed (mean \pm SD triglycerides 5.0 ± 6.7 mmol/L; cholesterol 7.1 ± 2.7 mmol/L).

The precipitation capacity of the various HDL-chol methods was studied in two ways. Initially, sera were analyzed after addition of increasing concentrations of saccharose (20, 75, 125, or 200 g/L) to produce samples characterized by an increase in background absorbance and a slight increase in viscosity. This addition interferes with the sedimentation of lipoproteins and mirrors the precipitation problems routinely seen in strongly lipemic sera. For more-definitive conclusions, we also compared the precipitation efficiency of the Tung-B, Dex, and PEG methods for analysis of frozen stored hypertriglyceridemic serum samples with triglycerides concentrations as great as 54.7 mmol/L (mean \pm SD plasma triglycerides 18.8 ± 12.0 mmol/L; plasma cholesterol 10.6 ± 2.8 mmol/L). After centrifugation of the incubated sample/reagent mixtures under routine conditions, we analyzed whether the supernate was clear, turbid, or clear with a lipid layer at the meniscus, as a function of the serum triglyceride concentration. These precipitation methods were selected for evaluation because they show the largest range in the final background density (specific gravity) according to variations in sample/reagent ratio (Tung-B vs Dex) or the most variation in coprecipitation of proteins (PEG and Tung-B vs Dex).

Separately, 10 to 34 similarly strongly lipemic sera were analyzed with the various precipitation methods; all had a plasma triglyceride concentration >10 mmol/L, and all were analyzed both undiluted and twofold diluted with saline or with bovine serum albumin (Cohn Fraction V), 40 g/L.

STATISTICAL ANALYSIS

Results are given as mean \pm SD and were analyzed by Student's paired t -test. Results obtained by different methods were correlated by use of Pearson's correlation test with the application of the SSPS/PC statistical software (version 3.1; SSPS, Chicago, IL). We also calculated the standard errors (SE) in the intercept, slope, and estimate ($S_{y|x}$), using the test of Passing and Bablock. For most intermethod comparisons, the deviations in intercept and slope did not deviate from the ideal curve $y = x$ [16]. This behavior is, therefore, not explicitly mentioned for each method in *Results*. Differences were considered significant if P was <0.05 .

Results

Accuracy of the various precipitation methods. After several trial sessions to become acquainted with the various precipitation procedures, the solo technician assayed 26 different sera over 2 days in the first part of this study. As in a previous evaluation in which we analyzed the samples for HDL with two different cholesterol reagents, we observed a good agreement between the results of all methods. On the basis of the previous study, we selected the PEG 6000 method as the comparison method. Evaluation of the results showed that those obtained with Tung-B, Dex, PEG 6000, and PEG/Dex methods were similar ($P > 0.4$; intermethod CV = 3.6%). Given the relatively small number of samples analyzed, we averaged the results obtained in the Tung-B, Dex, PEG 6000, and PEG/Dex methods and used these averages as "consensus" values with which the results of all HDL methods were compared. In agreement with previous results [9], the Hep results showed a positive bias, despite the blank correction (Table 2). This is inherent to (typical of) interference of Mn^{2+} with the cholesterol assay, not an inaccuracy of the precipitation procedure. Also in agreement with the previous study [9], the Tung-L method results showed a negative bias, for unknown reasons.

Precipitation capacity of the methods. As evaluated with the saccharose-containing samples and hypertriglyceridemic sera, both second-generation methods had the best precipitation efficiency, successfully fractionating samples with triglyceride concentrations of <16.4 to 17.6 mmol/L. The precipitation efficiency of the PEG method was slightly lower, followed in order by the Tung-L, Dex, and Hep methods. The supplier states that twofold dilution with saline enhances the precipitation efficiency. This was indeed true but gave slightly inaccurate results: 0.63 ± 0.32 mmol/L vs 0.72 ± 0.31 mmol/L in undiluted samples ($n = 10$, $P < 0.001$). The difference was smaller when 60 g/L bovine serum albumin instead of saline was used as diluent for turbid samples: 0.60 ± 0.22 vs 0.64 ± 0.32 mmol/L, respectively ($n = 34$, $P < 0.001$). This latter modification was indeed very effective, such that only sera with triglyceride concentrations >28.0 mmol/L needed ultrafiltration. Such samples necessarily contain chylomicrons but, by careful handling, aspiration of the flocculate at the meniscus can largely be avoided. Consequently, the obligatory ultrafiltration step is very simple to perform. Moreover, even without dilution, eventual ultrafiltration of these samples gave values similar to those obtained with the PEG method: 0.74 ± 0.28 vs 0.74 ± 0.31 mmol/L, respectively ($n = 34$).

Table 2. Results of 6 different HDL-chol precipitation methods compared.

HDL-chol methods compared (x)	Slope		Intercept, mmol/L		r	Mean x, mmol/L	$S_{x y}$ mmol/L
	Mean	95% CI	Mean	95% CI			
Heparin/ Mn^{2+}	1.03	0.91–1.17	0.08	–0.08 to 0.25	0.94	1.45 (0.35) ^a	0.07
Tung-L	0.97	0.93–1.03	–0.04	–0.11 to 0.02	1.00	1.21 (0.34) ^a	0.02
Tung-B	1.13	1.05–1.21	–0.12	–0.24 to –0.05	0.99	1.32 (0.39)	0.02
Dex	1.00	0.94–1.06	–0.02	–0.08 to 0.06	1.00	1.27 (0.34)	0.02
PEG 6000	1.08	0.98–1.15	–0.01	–0.11 to 0.10	0.99	1.37 (0.37)	0.03
PEG/Dex	1.05	0.94–1.07	–0.05	–0.06 to 0.04	0.98	1.30 (0.34)	0.02

Results obtained by one experienced technician for 26 sera with the various methods were compared with the consensus value [1.32 (0.35) mmol/L], calculated from the average results obtained with the Tung-B, Dex, PEG 6000, and PEG/Dex methods.

^a $P < 0.01$ by Student's *t*-test vs the consensus value.

Table 3. Precipitation efficiency of the various HDL-chol precipitation methods.

HDL not successfully precipitated at ^a

HDL method	Saccharose, g/L	Triglycerides, mmol/L	
		Present study	Former study [9]
Hep	>75	ND	>2.4
Tung-L	>125	ND	>7.6
Tung-B	>200	>16.4 to 17.6 (>28.0) ^b	ND
Dex	>75	>4.1	>4.0
PEG 6000	>125	>10.0	>18.5
PEG/Dex	>200	ND	ND

^a Precipitation was tested in two ways: by raising the background absorbance of the serum with saccharose (final concentrations 75, 125, or 200 g/L); and by assaying hypertriglyceridemic serum with triglyceride concentrations up to 54.7 mmol/L.

^b Concentration below which sedimentation of apolipoprotein B-containing lipoproteins was successful after diluting the serum with an equal volume of 60 g/L bovine serum albumin.

ND, not done.

Although we did not evaluate this possibility, the PEG/Dex method probably yields the lowest number of turbid supernates for these strongly lipemic samples because the samples are the most highly diluted in this method.

Imprecision of the various methods. Three frozen stored serum samples (cholesterol <6.0 mmol/L, triglycerides <2.0 mmol/L) were analyzed with the various HDL-chol procedures by an experienced technician on 5 different days. None of these sera yielded turbid supernates. The imprecision of the various methods, expressed as SDs, ranged from 0.02 ± 0.01 mmol/L to 0.04 ± 0.01 mmol/L. Analyses of the samples containing 200 g/L saccharose with the first-generation precipitation methods frequently required ultrafiltration of the supernates. Because of the ultrafiltration step, the imprecision in these samples with either precipitation reagent was similar to the values obtained for the frozen serum pools without saccharose, except for the values obtained with the Hep method ($SD\ 0.12 \pm 0.05$ mmol/L). Thus, both second-generation precipitation methods were the most efficient and resulted in the highest possible precision.

Evaluation of the Tung-B method under routine conditions. The Tung-B method appears to be accurate and precise, and its reagent composition is simple and widely available, in contrast to the PEG/Dex reagent. Therefore, we selected the Tung-B method for future use on the routine laboratory. As a test, 33 different technicians analyzed 406 samples over 6 weeks. The results agreed well with those obtained with the PEG method, irrespective of the triglyceride concentration or any need for ultrafiltration (Fig. 1).

Discussion

For the second time we show that most first-generation HDL-chol methods are similarly accurate, except for the Tung-L method, which in our hands has a slight negative bias. The positive bias we obtained for the Hep method resulted from interference of Mn^{2+} in cholesterol determination with the CHOD-PAP reagent. This interference is absent when other cholesterol reagents are used, but in this study necessitated a blank determination for each sample [6] (the catalase cholesterol reagent we used in our earlier study is no longer available). However, as suggested in our previous work, the PEG 6000 method, coupled to cholesterol analysis with the CHOD-PAP reagent, emerged as a reliable method suitable for comparison purposes because of (a) its excellent agreement with the ultracentrifugation/Hep precipitation method, (b) its good agreement with density-gradient ultracentrifugation [17], (c) its precipitation efficiency in lipemic samples, and (d) our positive experience with this method in all our research projects in the last 15 years. Moreover, calibrating the Tung-B method against the PEG 6000 method fulfilled National Cholesterol Education Program

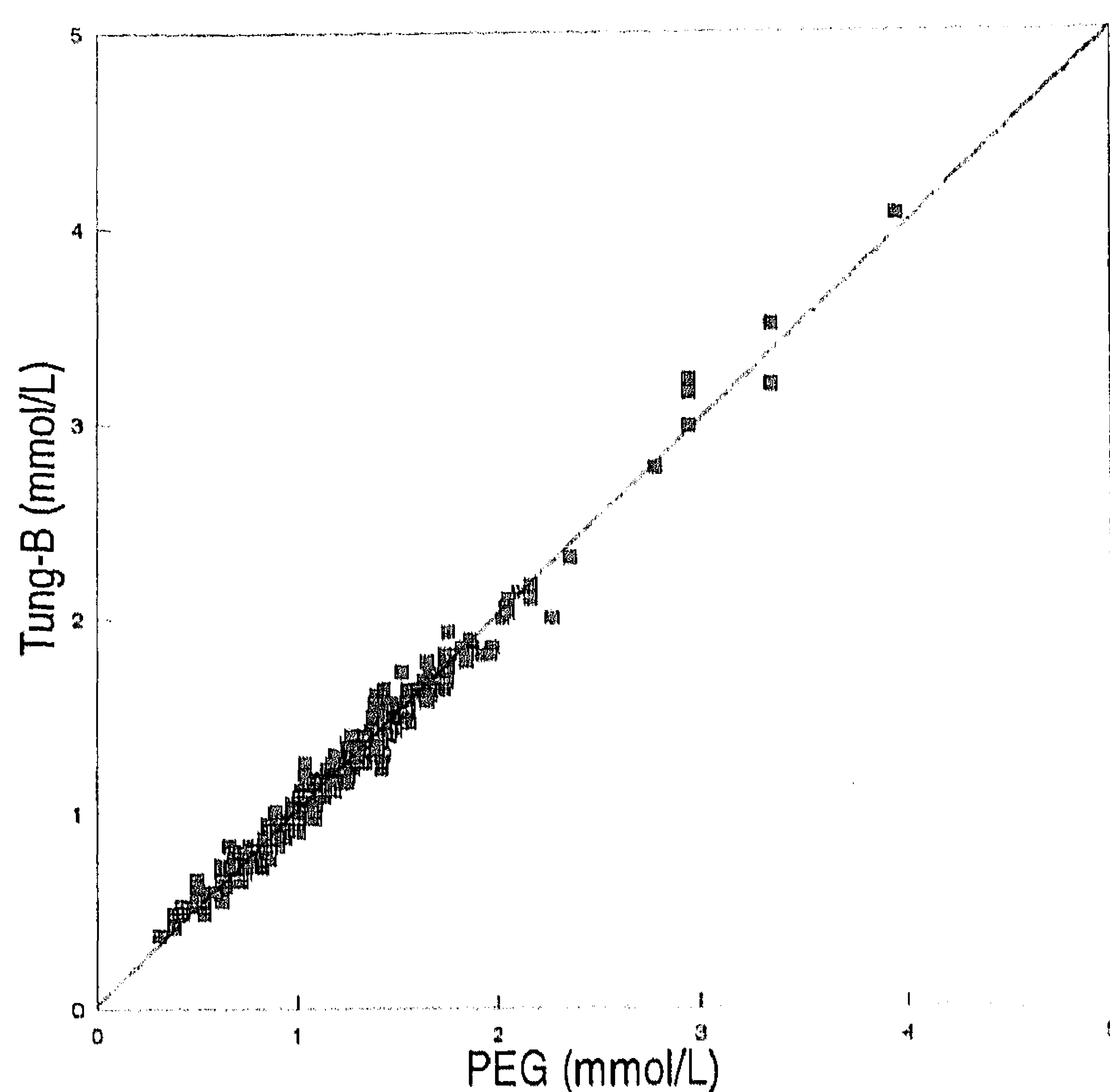


Fig. 1. HDL-chol values obtained with the Tung-B (y) and PEG (x) methods compared.

The Tung-B method was used under routine conditions with 33 participating technicians; the PEG method was performed at the research laboratory by several experienced technicians. As stated in the text, 406 samples (mean \pm SD triglycerides 5.0 ± 6.7 mmol/L; cholesterol 7.1 ± 2.7 mmol/L) were analyzed, giving the following results: $\bar{x} = 1.18 \pm 0.45$ mmol/L, $\bar{y} = 1.19 \pm 0.46$ mmol/L; linear regression equation: $y = (1.00 \pm 0.01)x + (0.00 \pm 0.01)$ mmol/L; $r = 0.98$, $S_{y/x} = 0.030$ mmol/L. In a separate series were analyzed 23 hyperlipidemic samples with triglycerides concentrations up to 10 mmol/L; these samples, which were twofold diluted with 40 g/L bovine serum albumin and eventually ultrafiltered, yielded the following results: $\bar{x} = 0.59 \pm 0.20$ mmol/L, $\bar{y} = 0.60 \pm 0.18$ mmol/L; linear regression equation: $y = (0.92 \pm 0.04)x + (0.04 \pm 0.06)$ mmol/L; $r = 0.96$, $S_{y/x} = 0.040$ mmol/L.

criteria for accuracy and precision in comparison with the CDC HDL-chol method.

The HDL-chol determination necessarily consists of two steps: separation of the apolipoprotein B-containing lipoproteins from the HDL particles, followed by accurate and precise determination of the cholesterol in the HDL fractions. For many years our research and routine laboratories have participated in a national quality program designed to control the quality of total serum cholesterol determinations. Control sera are targeted on the basis of analysis with the certified Abell-Kendall method performed at the Lipid Reference Laboratory at Rotterdam [18]. With this as a base for accuracy, we designed specific modifications so as to determine cholesterol at the low concentrations characteristic for HDL samples produced with either first- or second-generation precipitation methods. This involved simply increasing the ratio of sample to cholesterol reagent and diluting our primary calibrators. Irrespective of the absolute cholesterol concentration in the samples, precision was always satisfactory, at least if a proper sample/reagent ratio was selected.

Because of its greater precipitation efficiency, the Tung-B method is more generally applicable than the PEG 6000 method. The precipitation efficiency of the Dex method, a Proposed Selected Method and intended to be used as a "designated comparison method" in CDC

HDL-chol standardization, is limited, however. Therefore, we suggest a broader application of the Tung-B method for standardization purposes.

Recently, "third-generation" HDL-chol methods have been introduced, in which no precipitation step is used [19, 20]. Samples with triglyceride concentrations as great as 30 mmol/L can be analyzed without problems by using bichromatic analysis. Evaluations at higher triglyceride concentrations are scarce, although the results reported thus far are promising [19–22]. Such direct HDL analysis is efficient, but the reagent costs, including the disposable costs, are two- to threefold higher than with the Tung-B method. Moreover, the results by the Tung method are at least as good as those by the direct HDL method. The choice between both options is, therefore, a question of the motivation of the technicians and the available budget.

We thank C. Boersma Cobbaert (Lipid Reference Laboratory, Erasmus University, Rotterdam, The Netherlands) for performing the CDC HDL-chol analyses.

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We evaluated six precipitation methods for high-density lipoprotein cholesterol (HDL-chol) determination: the heparin/Mn²⁺ precipitation reagent method (Hep), two variants of the phosphotungstic acid/Mg²⁺ method (Tung-L and Tung-B), the dextran sulfate 50 000/Mg²⁺ method (Dex), the PEG 6000 method (PEG), and the PEG 6000/dextran sulfate 15 000 (PEG/Dex) method. The Tung-B and PEG/Dex precipitation methods have a low sample/precipitation reagent volume ratio (<0.4). The Tung-B, Dex, PEG, and PEG/Dex methods gave similar values, averaging within 0.1 mmol/L of each other, showing that the precipitation selectivity of these methods is comparable. The precipitation efficiency of Tung-B and PEG/Dex, however, was superior. Ultrafiltration of the supernatants was needed only at triglyceride concentrations >16.4 mmol/L (undiluted sample) or >28.0 mmol/L (sample diluted twofold); however, ultrafiltration without dilution was the most accurate method. Results of Tung-B under routine conditions (33 technicians) agreed well with those of the PEG method for 406 normo- and hyperlipidemic plasma samples. By comparison with the HDL-chol method from the Centers for Disease Control and Prevention, the Tung-B method showed a total error of 10.6%, which fulfills the criteria of the National Cholesterol Education Program for HDL-chol analysis. In conclusion, with motivated personnel, Tung-B is a reliable, cost-effective method for routine HDL-chol analysis.

INDEXING TERMS: lipoproteins • method comparison • laboratory management

Proper risk estimation for coronary heart disease involves total cholesterol and high-density lipoprotein cholesterol (HDL-chol) measurements [1-4].³ HDL-chol measurement is still problematic, despite the general use of a Proposed Selected Method [5], especially in samples that have been frozen and in lipemic plasma samples [6, 7]. Earlier, we introduced and fully validated the polyethylene glycol (PEG) 6000 method [8]. Given its excellent agreement with ultracentrifugation/heparin-Mn²⁺ (Hep) precipitation and its problem-free use in all our research projects in the last 15 years, the PEG 6000 method has, in our area, gradually gained the status of a "Regional Designated Comparison Method." This attainment was stimulated by its superior precipitation efficiency among all the first-generation precipitation methods [6, 9]. Although the results obtained with the PEG 6000 method are scientifically reliable, the analyses must be performed by a specialized group of technicians.

Given the steady growth in the number of requests for HDL-chol determinations, we felt the need for another method, one that could be used more generally, i.e., in the routine laboratory also. For this purpose we evaluated several "first-generation" precipitation methods, includ-

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Received June 5, 1996; revised November 14, 1996; accepted November 15, 1996.

³ Nonstandard abbreviations: chol, cholesterol; Hep, heparin/Mn²⁺; Tung-L, phosphotungstate/Mg²⁺ (Lopes-Virella version); Tung-B, phosphotungstate/Mg²⁺ (Boehringer version); Dex, dextran sulfate 50 000/Mg²⁺; PEG 6000, polyethylene glycol 6000; PEG/Dex, polyethylene glycol 6000/dextran sulfate 15 000; first- (second-) generation precipitation method, HDL assay performed with concentrated (diluted) precipitation reagent, resulting in concentrated (diluted) HDL samples; macro- and micromethods, cholesterol analysis under conditions optimal for cholesterol concentrations between 0.1 and 3.5 mmol/L or from 0.01 to 0.35 mmol/L, respectively; CDC, Centers for Disease Control and Prevention.

ing the Proposed Selected Method, which uses dextran sulfate 50 000/Mg²⁺ (Dex) [5]. We also included two "second-generation" precipitation methods, which use a more diluted precipitation reagent to obtain more-effective precipitation. In this study we clearly show that not only methodological but also personnel aspects must be taken into account in generating the best analytical results in a routine laboratory.

Materials and Methods

PROCEDURES, PART 1

In the first part of this study, the merits of six different precipitation methods were compared by a single experienced technician. The first-generation procedures used are listed here in the same order as in our previous study [9]. In that study these procedures were validated and compared against an ultracentrifugation/precipitation procedure, very similar to the Centers for Disease Control and Prevention (CDC) HDL method, except that the cholesterol was determined with enzymatic analysis. In these first-generation HDL methods, the volume of serum is >5 times the volume of precipitation reagent. In Tung-B and PEG/Dex, second-generation methods, the volume of serum is <0.4 times the volume of precipitation reagent. In the first part of the study, in which only a limited number of samples were analyzed, the results were compared with consensus values, i.e., the average values obtained with four different methods that appeared to give very similar results. Assay conditions of the various precipitation methods, and of the different ways we analyzed for cholesterol, are summarized in Table 1.

In the first part of this study, the following precipitation procedures were evaluated:

Hep [9, 10]. One milliliter of serum was mixed with 100 μ L of a mixture of equal parts of 40 g/L sodium heparinate solution (156 USP units/mg; Organon, Oss, The Netherlands) and 1 mol/L MnCl₂ solution. After 10 min, the samples were centrifuged for 15 min at 4000g. The supernate was aspirated as described [9] with a Pasteur pipette. When turbid, the supernatant was filtered through a 0.20- μ m (average pore size) filter (FP030/3; Schleicher and Schüll, Dassel, Germany) [9]. Cholesterol content was quantified with the macromethod described in Table 1, and the final concentration was corrected for dilution.

Tung-L method: phosphotungstate/Mg²⁺ [9–11]. Optimized phosphotungstate reagent was prepared by dissolving 40 g of phosphotungstic acid (cat. no. 583; Merck, Darmstadt, Germany) in distilled water. The pH of the solution was adjusted to 6.15 with 1 mol/L NaOH [12], and the reagent was diluted to 1 L with water. The Mg²⁺ concentration of the 2.5 mol/L MgCl₂ solution was checked by atomic absorption spectrometry [13]. One milliliter of serum was mixed with 20 μ L of the 2.5 mmol/L MgCl₂ solution (final concentration: 1.39 μ mol of phosphotungstic acid and 50 μ mol of MgCl₂ per milliliter of serum).

Table 1. Procedures used in the two parts of this study.

First part		Second part	
Precipitation methods	Cholesterol method	Precipitation methods	Cholesterol method
Heparin/Mn ²⁺	Macro ^a		
Phosphotungstate/Mg ²⁺ (Tung-L)	Macro ^a		
Phosphotungstate/Mg ²⁺ (Tung-B)	Micro ^b	Tung-B	Micro ^c
Dextran sulfate/Mg ²⁺	Macro ^a		
PEG 6000	Macro ^a	PEG 6000	Macro ^a
PEG/Dex	Micro ^b		

^a Macro method on the Multistat IL-III analyzer with the use of CHOD-PAP reagent (Boehringer Mannheim, cat. no. 237574). Sample/reagent volumes (μ L): 5/150, diluted further with 45 μ L of rinsing water; calibrated with Preciset solution diluted with Brij-35 wetting agent (no. 430AG-6; Sigma Chemical Co., St. Louis, MO) to final cholesterol concentrations of 0.08, 0.39, and 1.29 mmol/L. The method for total cholesterol measurement regularly checked for accuracy against the CDC-certified Abell-Kendall method (Lipid Reference Laboratory, Rotterdam) in the framework of a specific Dutch cholesterol standardization program gave observed biases <1.2%.

^b Micro method on the Multistat IL-III analyzer as above but with sample/reagent volumes (μ L) of 80/80 further diluted with 20 μ L of rinsing water; calibrated with Preciset diluted with Brij to final cholesterol concentrations of 0.130 and 0.388 mmol/L.

^c Micro analysis on the Hitachi 747 analyzer with CHOD-PAP reagent (cat. no. 1489704). Sample/reagent volumes (μ L): 20/250; calibrated with calibrating serum (Boehringer, cat. no. 759350) and the specific Hitachi calibrating procedure. The method is regularly checked for accuracy of total cholesterol measurement against the CDC-certified Abell-Kendall procedure as mentioned above; observed biases thus far have been <1.3%. Imprecision = 1.9% (n = 30). To attain this accuracy, we routinely corrected the target value of the calibrating serum (provided by the supplier) by -4%.

After 15 min, the HDL fraction was isolated as described, and cholesterol was determined with the macromethod.

Tung-B method: phosphotungstate/Mg²⁺. We also applied a second version of the phosphotungstate/Mg²⁺ method, using reagent from Boehringer Mannheim (Mannheim, Germany; cat. no. 543004): 200 μ L of serum was mixed with 500 μ L of precipitation reagent (final concentrations: 1.1 μ mol of phosphotungstic acid and 50 mmol of MgCl₂ per milliliter of serum). After incubation, the supernate was isolated as described above, and the cholesterol was determined with a microassay (see Table 1).

Dex [5]. We used reagent obtained from Johnson & Johnson (Rochester, NY), pipetting 0.5 mL of serum into the Ektachem (Johnson & Johnson) HDL assay tube containing dextran sulfate 50 000/magnesium reagent. After incubation, we applied the conditions for fractionation and cholesterol analysis described above. One milliliter of serum was mixed with 100 μ L of a mixture of equal parts of 2 mol/L MgCl₂ and dextran sulfate; the HDL fraction was isolated as described and assayed for cholesterol with the macromethod.

PEG 6000 [6, 8]. One milliliter of serum was mixed with 200 μ L of PEG 6000 solution [22.5 g of Merck no. 807491

and 22.5 g of Fluka (Fuchs, Switzerland) no. 81260, both dissolved in 100 mL of 0.2 mol/L Tris-HCl buffer, pH 8.2]. The final PEG 6000 concentration in serum was thus 75 g/L. After thorough vortex-mixing, incubation, and centrifugation, HDL fractions were isolated as above and were determined for cholesterol with the macromethod.

PEG/Dex [14]. The reagent, from Instruchemie (Hilverum, The Netherlands), contained 0.15 mol/L NaCl, 100 g/L PEG 6000, 37.4 mg/L dextran sulfate 15 000, and 2.6 mmol/L MgCl_2 . Serum (100 μL) was mixed with 1000 μL of the HDL-chol precipitation reagent. After incubation, the supernate was isolated and cholesterol was assayed with the micromethod.

Cholesterol quantification. The cholesterol in the supernates was determined with a Multistat IL-III (Instrumentation Laboratory, Lexington, MA) centrifugal analyzer by one of two methods, depending on the final concentration of the analyte in the sample after dilution (Table 1). We used the CHOD-PAP reagent (cat. no. 237574; Boehringer Mannheim). The results of both the micro- and the macro-version assays (for 1.2- and 11-fold-diluted plasma samples, respectively) showed good agreement ($r = 0.999$). In all measurements, the actual cholesterol concentration found for the HDL fractions was corrected for the "precipitation blank" (apparent cholesterol concentration of appropriately diluted precipitation reagent); this generally amounted to <0.03 mmol/L, except for the Hep reagent, which gave a value of 0.10–0.15 mmol/L.

Triglycerides assay. Triglycerides were analyzed on the Hitachi 747 analyzer with reagent no. 1361155 (both from Boehringer Mannheim). We checked the method's accuracy against that of a semiautomated colorimetric method [15]. Imprecision was 1.6% ($n = 30$) for concentrations ≤ 12 mmol/L.

PROCEDURES, PART 2

In the second part of this study, the Tung-B method was evaluated under routine conditions by 33 different technicians in the routine laboratory, the technicians having been individually instructed by the one experienced colleague who performed the first part of the study. Because of the extensive previous validation and experience with the PEG 6000 method, we used this method for comparison. Cholesterol was measured in the routine laboratory with a Hitachi 747 analyzer (using the microassay version described in Table 1). As a further validation of the Tung-B method, we analyzed for 3 days in duplicate 7 fresh serum samples with HDL-chol concentrations ranging from 0.73 to 2.13 mmol/L; aliquots of these sera were also analyzed by the official CDC HDL-chol method at the Lipid Reference Laboratory (Rotterdam, The Netherlands) under the supervision of C. Cobbaert-Boersma.

SPECIMENS

Blood samples from normolipidemic subjects and from patients with various types of hyperlipoproteinemia were drawn into Vacutainer Tubes (Becton Dickinson, Rutherford, NJ). Serum was isolated within 2 h and was stored at 4 °C for no more than 2 days. In the first part of the study, 26 fresh serum samples with triglycerides <4.7 mmol/L were analyzed. In the second part, 406 sera or EDTA-containing plasma samples were analyzed (mean \pm SD triglycerides 5.0 ± 6.7 mmol/L; cholesterol 7.1 ± 2.7 mmol/L).

The precipitation capacity of the various HDL-chol methods was studied in two ways. Initially, sera were analyzed after addition of increasing concentrations of saccharose (20, 75, 125, or 200 g/L) to produce samples characterized by an increase in background absorbance and a slight increase in viscosity. This addition interferes with the sedimentation of lipoproteins and mirrors the precipitation problems routinely seen in strongly lipemic sera. For more-definitive conclusions, we also compared the precipitation efficiency of the Tung-B, Dex, and PEG methods for analysis of frozen stored hypertriglyceridemic serum samples with triglycerides concentrations as great as 54.7 mmol/L (mean \pm SD plasma triglycerides 18.8 ± 12.0 mmol/L; plasma cholesterol 10.6 ± 2.8 mmol/L). After centrifugation of the incubated sample/reagent mixtures under routine conditions, we analyzed whether the supernate was clear, turbid, or clear with a lipid layer at the meniscus, as a function of the serum triglyceride concentration. These precipitation methods were selected for evaluation because they show the largest range in the final background density (specific gravity) according to variations in sample/reagent ratio (Tung-B vs Dex) or the most variation in coprecipitation of proteins (PEG and Tung-B vs Dex).

Separately, 10 to 34 similarly strongly lipemic sera were analyzed with the various precipitation methods; all had a plasma triglyceride concentration >10 mmol/L, and all were analyzed both undiluted and twofold diluted with saline or with bovine serum albumin (Cohn Fraction V), 40 g/L.

STATISTICAL ANALYSIS

Results are given as mean \pm SD and were analyzed by Student's paired t -test. Results obtained by different methods were correlated by use of Pearson's correlation test with the application of the SSPS/PC statistical software (version 3.1; SSPS, Chicago, IL). We also calculated the standard errors (SE) in the intercept, slope, and estimate ($S_{y|x}$), using the test of Passing and Bablock. For most intermethod comparisons, the deviations in intercept and slope did not deviate from the ideal curve $y = x$ [16]. This behavior is, therefore, not explicitly mentioned for each method in *Results*. Differences were considered significant if P was <0.05 .

Results

Accuracy of the various precipitation methods. After several trial sessions to become acquainted with the various precipitation procedures, the solo technician assayed 26 different sera over 2 days in the first part of this study. As in a previous evaluation in which we analyzed the samples for HDL with two different cholesterol reagents, we observed a good agreement between the results of all methods. On the basis of the previous study, we selected the PEG 6000 method as the comparison method. Evaluation of the results showed that those obtained with Tung-B, Dex, PEG 6000, and PEG/Dex methods were similar ($P > 0.4$; intermethod CV = 3.6%). Given the relatively small number of samples analyzed, we averaged the results obtained in the Tung-B, Dex, PEG 6000, and PEG/Dex methods and used these averages as "consensus" values with which the results of all HDL methods were compared. In agreement with previous results [9], the Hep results showed a positive bias, despite the blank correction (Table 2). This is inherent to (typical of) interference of Mn^{2+} with the cholesterol assay, not an inaccuracy of the precipitation procedure. Also in agreement with the previous study [9], the Tung-L method results showed a negative bias, for unknown reasons.

Precipitation capacity of the methods. As evaluated with the saccharose-containing samples and hypertriglyceridemic sera, both second-generation methods had the best precipitation efficiency, successfully fractionating samples with triglyceride concentrations of <16.4 to 17.6 mmol/L. The precipitation efficiency of the PEG method was slightly lower, followed in order by the Tung-L, Dex, and Hep methods. The supplier states that twofold dilution with saline enhances the precipitation efficiency. This was indeed true but gave slightly inaccurate results: 0.63 ± 0.32 mmol/L vs 0.72 ± 0.31 mmol/L in undiluted samples ($n = 10$, $P < 0.001$). The difference was smaller when 60 g/L bovine serum albumin instead of saline was used as diluent for turbid samples: 0.60 ± 0.22 vs 0.64 ± 0.32 mmol/L, respectively ($n = 34$, $P < 0.001$). This latter modification was indeed very effective, such that only sera with triglyceride concentrations >28.0 mmol/L needed ultrafiltration. Such samples necessarily contain chylomicrons but, by careful handling, aspiration of the flocculate at the meniscus can largely be avoided. Consequently, the obligatory ultrafiltration step is very simple to perform. Moreover, even without dilution, eventual ultrafiltration of these samples gave values similar to those obtained with the PEG method: 0.74 ± 0.28 vs 0.74 ± 0.31 mmol/L, respectively ($n = 34$).

Table 2. Results of 6 different HDL-chol precipitation methods compared.

HDL-chol methods compared (x)	Slope		Intercept, mmol/L		r	Mean x, mmol/L	$S_{x y}$ mmol/L
	Mean	95% CI	Mean	95% CI			
Heparin/ Mn^{2+}	1.03	0.91–1.17	0.08	–0.08 to 0.25	0.94	1.45 (0.35) ^a	0.07
Tung-L	0.97	0.93–1.03	–0.04	–0.11 to 0.02	1.00	1.21 (0.34) ^a	0.02
Tung-B	1.13	1.05–1.21	–0.12	–0.24 to –0.05	0.99	1.32 (0.39)	0.02
Dex	1.00	0.94–1.06	–0.02	–0.08 to 0.06	1.00	1.27 (0.34)	0.02
PEG 6000	1.08	0.98–1.15	–0.01	–0.11 to 0.10	0.99	1.37 (0.37)	0.03
PEG/Dex	1.05	0.94–1.07	–0.05	–0.06 to 0.04	0.98	1.30 (0.34)	0.02

Results obtained by one experienced technician for 26 sera with the various methods were compared with the consensus value [1.32 (0.35) mmol/L], calculated from the average results obtained with the Tung-B, Dex, PEG 6000, and PEG/Dex methods.

^a $P < 0.01$ by Student's *t*-test vs the consensus value.

Table 3. Precipitation efficiency of the various HDL-chol precipitation methods.

HDL not successfully precipitated at ^a

HDL method	Saccharose, g/L	Triglycerides, mmol/L	
		Present study	Former study [9]
Hep	>75	ND	>2.4
Tung-L	>125	ND	>7.6
Tung-B	>200	>16.4 to 17.6 (>28.0) ^b	ND
Dex	>75	>4.1	>4.0
PEG 6000	>125	>10.0	>18.5
PEG/Dex	>200	ND	ND

^a Precipitation was tested in two ways: by raising the background absorbance of the serum with saccharose (final concentrations 75, 125, or 200 g/L); and by assaying hypertriglyceridemic serum with triglyceride concentrations up to 54.7 mmol/L.

^b Concentration below which sedimentation of apolipoprotein B-containing lipoproteins was successful after diluting the serum with an equal volume of 60 g/L bovine serum albumin.

ND, not done.

Although we did not evaluate this possibility, the PEG/Dex method probably yields the lowest number of turbid supernates for these strongly lipemic samples because the samples are the most highly diluted in this method.

Imprecision of the various methods. Three frozen stored serum samples (cholesterol <6.0 mmol/L, triglycerides <2.0 mmol/L) were analyzed with the various HDL-chol procedures by an experienced technician on 5 different days. None of these sera yielded turbid supernates. The imprecision of the various methods, expressed as SDs, ranged from 0.02 ± 0.01 mmol/L to 0.04 ± 0.01 mmol/L. Analyses of the samples containing 200 g/L saccharose with the first-generation precipitation methods frequently required ultrafiltration of the supernates. Because of the ultrafiltration step, the imprecision in these samples with either precipitation reagent was similar to the values obtained for the frozen serum pools without saccharose, except for the values obtained with the Hep method ($SD\ 0.12 \pm 0.05$ mmol/L). Thus, both second-generation precipitation methods were the most efficient and resulted in the highest possible precision.

Evaluation of the Tung-B method under routine conditions. The Tung-B method appears to be accurate and precise, and its reagent composition is simple and widely available, in contrast to the PEG/Dex reagent. Therefore, we selected the Tung-B method for future use on the routine laboratory. As a test, 33 different technicians analyzed 406 samples over 6 weeks. The results agreed well with those obtained with the PEG method, irrespective of the triglyceride concentration or any need for ultrafiltration (Fig. 1).

Discussion

For the second time we show that most first-generation HDL-chol methods are similarly accurate, except for the Tung-L method, which in our hands has a slight negative bias. The positive bias we obtained for the Hep method resulted from interference of Mn^{2+} in cholesterol determination with the CHOD-PAP reagent. This interference is absent when other cholesterol reagents are used, but in this study necessitated a blank determination for each sample [6] (the catalase cholesterol reagent we used in our earlier study is no longer available). However, as suggested in our previous work, the PEG 6000 method, coupled to cholesterol analysis with the CHOD-PAP reagent, emerged as a reliable method suitable for comparison purposes because of (a) its excellent agreement with the ultracentrifugation/Hep precipitation method, (b) its good agreement with density-gradient ultracentrifugation [17], (c) its precipitation efficiency in lipemic samples, and (d) our positive experience with this method in all our research projects in the last 15 years. Moreover, calibrating the Tung-B method against the PEG 6000 method fulfilled National Cholesterol Education Program

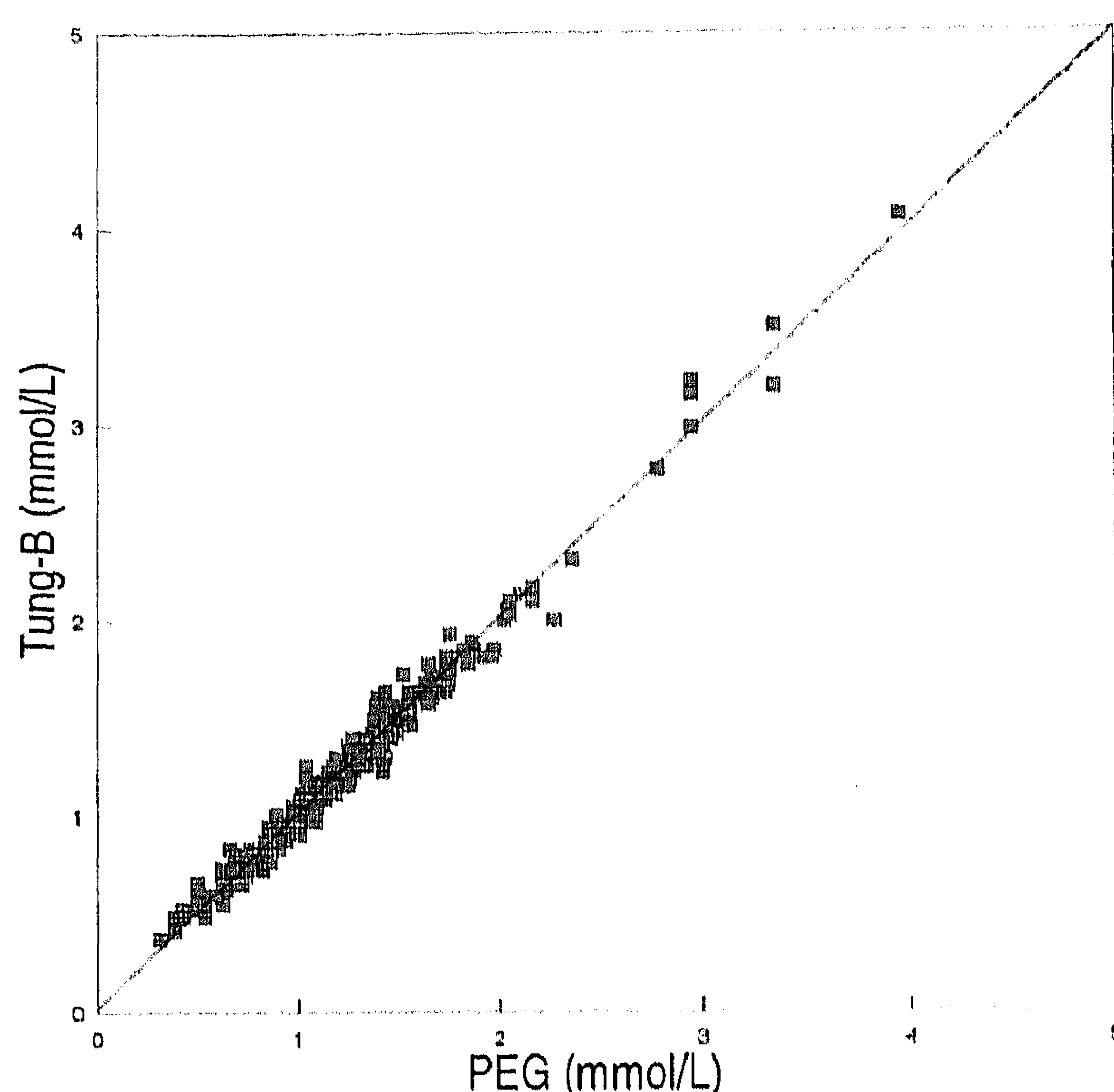


Fig. 1. HDL-chol values obtained with the Tung-B (y) and PEG (x) methods compared.

The Tung-B method was used under routine conditions with 33 participating technicians; the PEG method was performed at the research laboratory by several experienced technicians. As stated in the text, 406 samples (mean \pm SD triglycerides 5.0 ± 6.7 mmol/L; cholesterol 7.1 ± 2.7 mmol/L) were analyzed, giving the following results: $\bar{x} = 1.18 \pm 0.45$ mmol/L, $\bar{y} = 1.19 \pm 0.46$ mmol/L; linear regression equation: $y = (1.00 \pm 0.01)x + (0.00 \pm 0.01)$ mmol/L; $r = 0.98$, $S_{y/x} = 0.030$ mmol/L. In a separate series were analyzed 23 hyperlipidemic samples with triglycerides concentrations up to 10 mmol/L; these samples, which were twofold diluted with 40 g/L bovine serum albumin and eventually ultrafiltered, yielded the following results: $\bar{x} = 0.59 \pm 0.20$ mmol/L, $\bar{y} = 0.60 \pm 0.18$ mmol/L; linear regression equation: $y = (0.92 \pm 0.04)x + (0.04 \pm 0.06)$ mmol/L; $r = 0.96$, $S_{y/x} = 0.040$ mmol/L.

criteria for accuracy and precision in comparison with the CDC HDL-chol method.

The HDL-chol determination necessarily consists of two steps: separation of the apolipoprotein B-containing lipoproteins from the HDL particles, followed by accurate and precise determination of the cholesterol in the HDL fractions. For many years our research and routine laboratories have participated in a national quality program designed to control the quality of total serum cholesterol determinations. Control sera are targeted on the basis of analysis with the certified Abell-Kendall method performed at the Lipid Reference Laboratory at Rotterdam [18]. With this as a base for accuracy, we designed specific modifications so as to determine cholesterol at the low concentrations characteristic for HDL samples produced with either first- or second-generation precipitation methods. This involved simply increasing the ratio of sample to cholesterol reagent and diluting our primary calibrators. Irrespective of the absolute cholesterol concentration in the samples, precision was always satisfactory, at least if a proper sample/reagent ratio was selected.

Because of its greater precipitation efficiency, the Tung-B method is more generally applicable than the PEG 6000 method. The precipitation efficiency of the Dex method, a Proposed Selected Method and intended to be used as a "designated comparison method" in CDC

HDL-chol standardization, is limited, however. Therefore, we suggest a broader application of the Tung-B method for standardization purposes.

Recently, "third-generation" HDL-chol methods have been introduced, in which no precipitation step is used [19, 20]. Samples with triglyceride concentrations as great as 30 mmol/L can be analyzed without problems by using bichromatic analysis. Evaluations at higher triglyceride concentrations are scarce, although the results reported thus far are promising [19–22]. Such direct HDL analysis is efficient, but the reagent costs, including the disposable costs, are two- to threefold higher than with the Tung-B method. Moreover, the results by the Tung method are at least as good as those by the direct HDL method. The choice between both options is, therefore, a question of the motivation of the technicians and the available budget.

We thank C. Boersma Cobbaert (Lipid Reference Laboratory, Erasmus University, Rotterdam, The Netherlands) for performing the CDC HDL-chol analyses.

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